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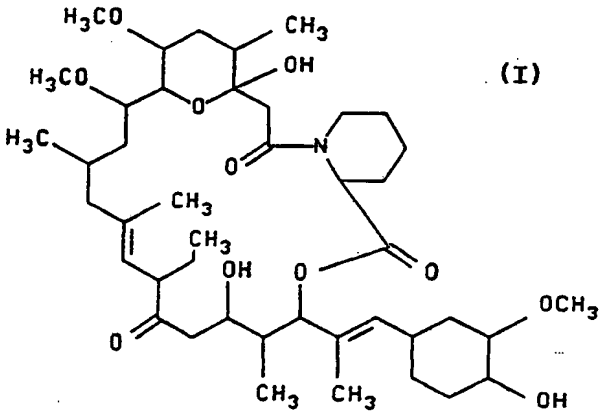
(54) Title: NOVEL IMMUNOSUPPRESSANT AGENT FROM STREPTOMYCES BRAEGENSIS



(I)

(57) Abstract

A compound of formula (I) is a novel immunosuppressant agent prepared by fermenting *Steptomyces braegensis* subsp. *pulcherrimus*, ATCC 55150, or another compound of formula (I) producing strain and extracting the compound of formula (I) from the fermentation medium. The compound is useful in treating transplant rejection and autoimmune diseases.

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NOVEL IMMUNOSUPPRESSANT AGENT FROM STREPTOMYCES BRAEGENSIS

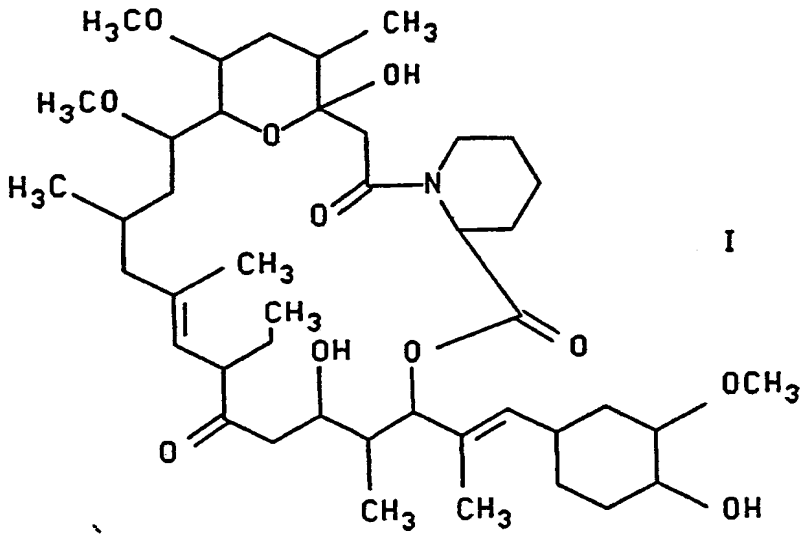
Background of the Invention

This invention relates to a new macrocyclic lactone immunosuppressant agent, a process for its production and its use in a human host for treatment of autoimmune diseases and/or prevention of organ transplant rejections. This application is a continuation of United States application 683,639 first filed on April 11, 1991. Priority is hereby claimed to that application.

In 1983, the United States Food and Drug Administration licensed cyclosporin, an anti-rejection drug that revolutionized the field of organ transplant surgery. The drug acts by inhibiting the body's immune system from mobilizing its vast arsenal of natural protecting agents to reject the transplant's foreign protein. Although cyclosporin is effective in fighting transplantation rejection, it suffers drawbacks in causing kidney failure, liver damage and ulcers which in many cases can be very severe. Newer, safer drugs exhibiting less side effects are constantly being searched for. European Patent Publication No. 0184162 of Fujisawa Pharmaceutical Co., Ltd., describes the macrolide immunosuppressants FK-506 and FK-520. The latter is produced by S. hygroscopicus subsp. yakushimaensis No. 7238. Other immunosuppressants are described in European Patent Application Publication Numbers 0323042, 0323865, 0356399 of Fisons plc, Merck & Co. Inc., and Sandoz, respectively.

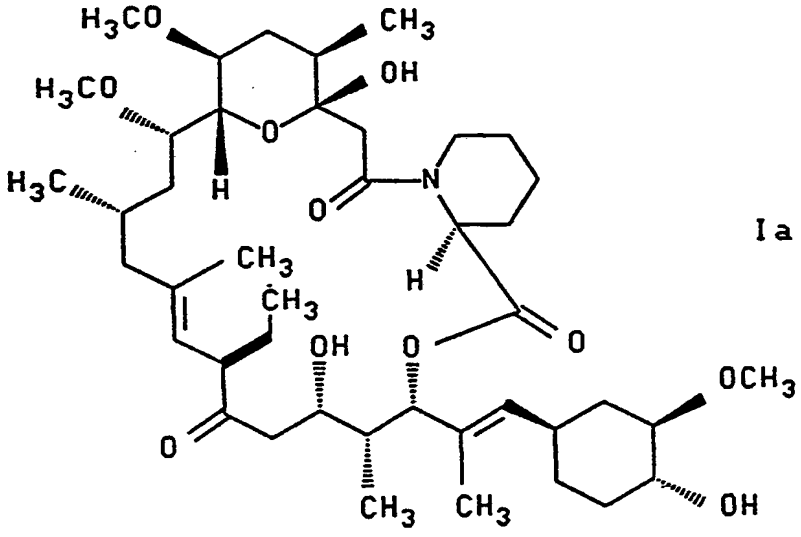
Summary of the Invention

The present invention relates to a new immunosuppressant of the formula



I

The compound of the formula I may be referred to as "C_9-desoxo-FK-520" because it differs from FK-520 in lacking an oxo group at the C_9 position. In view of the compound's similarity to FK-520 its stereochemistry is expected to be as shown in the following formula



Ia

The present invention also relates to a process for preparing the compound of the formula I comprising fermenting a compound of the formula I producing strain of bacteria (e.g., a Streptomyces strain such as Streptomyces

braegensis subsp. pulcherrimus Huang subsp. nov.) in an aqueous nutrient medium containing an assimilable carbon source and an assimilable nitrogen source, said fermentation being conducted preferably at a pH between about 4.0 and about 8.0, and extracting the compound of the formula I from the medium, preferably at a pH of about 4.0 to about 8.0.

The present invention also relates to a pharmaceutical composition containing an amount of the compound of the formula I effective in treating autoimmune disease (e.g. rheumatoid arthritis) or preventing organ transplant rejection in a mammal (e.g. a human) in combination with a pharmaceutically acceptable carrier.

The present invention also relates to a method of treating a mammal (e.g. a human) to prevent transplantation rejection or treating an autoimmune disease (e.g. rheumatoid arthritis) in a mammal (e.g. a human) comprising administering to said mammal a therapeutically effective amount of the compound of the formula I.

Finally, the present invention is directed to a biologically pure culture having the characteristics of Streptomyces braegensis subsp. pulcherrimus Huang subsp. nov., ATCC 55150, and ATCC 55150 per se, as well as mutants and transformants of any of the foregoing capable of producing the compounds of the formula I, including any such culture in freeze-dried form. Such a culture is capable of producing the compound of the formula I in a recoverable quantity upon fermentation in an aqueous nutrient medium comprising assimilable sources of carbon and nitrogen.

Description of the Drawings

Figure 1 is the proton NMR spectrum in $CDCl_3$ of the compound of the formula I, C_9-desoxo-FK-520.

Figure 2 is the carbon NMR spectrum in $CDCl_3$ of C_9-desoxo-FK-520.

Detailed Description of the Invention

In general, the compound of the formula I can be produced by culturing a compound of the formula I producing strain of bacteria in an aqueous nutrient medium containing

sources of assimilable carbon and nitrogen, preferably under submerged aerobic conditions (e.g. shaking culture, submerged culture, etc.). The aqueous medium is preferably maintained at a pH of about 4.0 to about 8.0 throughout the fermentation process. A higher pH may lead to loss of product. The desired pH may be maintained by the use of appropriate buffering agents.

A preferred culture for producing the compound of the formula I is designated Streptomyces braegensis subsp. pulcherrimus Huang subsp. nov., and has been deposited in The American Type Culture Collection, Rockville, Maryland under their accession number ATCC 55150.

This novel culture was derived from a soil sample collected in Misumitown, Yamaguchi prefecture, Japan, and identified in the culture collection of Pfizer Inc as N927-101. Its single colony isolate is identified as N927-101-SC50. Its description and classification were provided by Dr. L. H. Huang.

The cultures were found to produce narrow hyphae of the Actinomycetales, an unfragmented substrate mycelium, and an aerial mycelium on which spore chains are produced. The results of whole-cell analysis further indicates their belonging to the genus Streptomyces.

Each of culture N927-101 and culture N927-101SC50 was planted from a slant into ATCC #172 broth and grown for four days at 28°C on a shaker. It was then centrifuged for 20 minutes, washed three times with sterile water and planted on media commonly used for identification of members of the Actinomycetales.

The cultures were incubated at 28°C and the results were read at varying times but most commonly were taken at 14 days. The colors were described in common terminology, but exact colors were determined by comparisons with color chips from the Color Harmony Manual, fourth edition. The methods of whole-cell amino acid and sugar analyses are those described in B. Becker et al., Appl. Microbiol., 12, 421-423 (1964); and in M.P. Lechevalier, J. Lab. Clin. Med.,

71, 934-944 (1968). For comparison purposes, the type strains of Streptomyces tsukubaensis BP-927, S. hygroscopicus subsp. yakushimaensis BP-928, S. nigrescens ATCC 23941, S. braegensis NRRL 12567, and S. braegensis subsp. japonicus N617-29 were used.

Identification media used for the characterization of the cultures and references for their composition are as follows:

1. Tryptone-Yeast Extract Broth - (ISP #1 medium, Difco).
2. Yeast Extract-Malt Extract Agar - (ISP #2 medium, Difco).
3. Oatmeal Agar - (ISP #3 medium, Difco).
4. Inorganic Salts-Starch Agar - (ISP #4 medium, Difco).
5. Glycerol-Asparagine Agar - (ISP #5 medium, Difco).
6. Peptone-Yeast Extract Iron Agar - (ISP #6 medium, Difco).
7. Czapek-Sucrose Agar - S.A. Waksman, The Actinomycetes, Vol. 2, medium no. 1, p. 328, (1961).
8. Glucose-Asparagine Agar - Ibid, medium no. 2, p. 328.
9. Bennett's Agar - Ibid, medium no. 30, p. 331.
10. Emerson's Agar - Ibid, medium no. 28, p. 331.
11. Nutrient Agar - Ibid, medium no. 14, p. 330.
12. Gordon and Smith's Tyrosine Agar - R.E. Gordon and M. M. Smith, J. Bacteriol., 69, 147-150 (1955).
13. Casein Agar - Ibid.
14. Calcium Malate Agar - S.A. Waksman, Bacteriol. Rev., 21, 1-29 (1957).
15. Gelatin - R. E. Gordon and J. M. Mihm, J. Bacteriol., 73, 15-27 (1957).
16. Starch - Ibid.
17. Organic Nitrate Broth - Ibid.
18. Dextrose Nitrate Broth - S.A. Waksman, The Actinomycetes, Vol. 2, medium no. 1, p. 328 (1961), with 3 g dextrose substituted for 30 g sucrose and agar omitted.

19. Potato Carrot agar - M.P. Lechevalier, J. Lab. and Clinical M..., 71, 934-944 (1968), but use only 30 g potatoes, 2.5 g carrots and 20 g agar.
20. 2% Tap Water Agar.
21. Skim Milk - Difco.
22. Cellulose utilization -
    a) H. L. Jensen, Proc. Linn. Soc. N.S.W., 55, 231-248 (1930)
    b) M. Levine and H. W. Schoenlein, A Compilation of Culture Media, medium no. 2511 (1930).
23. Carbohydrate Utilization - (ISP #9 medium, Difco).
24. Temperature Range - ATCC 172 medium in ATCC Media Handbook, 1st Ed., p. 10 (1984).

The cultural characteristics of culture N927-101SC50, which produced higher titers of the compound of the formula I than culture N927-101, are as follows:

Yeast Extract-Malt Extract Agar - Growth good; white, pale gray, pale pink-gray to pink-gray (near gray series 3 dc, 5 dc, 5 fe); raised; smooth, granular to wrinkled; aerial mycelium same as surface; reverse yellowish to dark brown (2 ic, 4 ni, 4 li); no soluble pigment.

Oatmeal Agar - Growth moderate to good; white, pale gray to gray (near gray series 3 dc, 3 fe); slightly raised, smooth to granular, aerial mycelium same as surface; reverse gray to pink-gray (near gray series 3 fe, 5 fe, 7 ih); soluble pigment pink-gray (near gray series 5 dc).

Inorganic Salts-Starch Agar - Growth moderate to good; white, pale gray, gray to pink-gray (near gray series 3 dc, 3 fe, 5 fe); raised, smooth to wrinkled, aerial mycelium same as surface; reverse purplish black (near gray series 7 ml); soluble pigment yellowish brown to pink (3 gc, 6 ca).

Glycerol-Asparagine Agar - Growth poor to moderate; white, cream, pale gray to gray (2 ca, near gray series 3 dc, 3 fe); appearing as isolated colonies, thin to slightly raised, smooth to granular; aerial mycelium white, pale gray to gray; reverse cream to yellowish (2 ca, 2 nc, 2 pe); no soluble pigment.

Czapek-Sucrose Agar - Growth poor to moderate, pale gray to gray (near gray series 3 dc, 3 fe, 5 ih); thin to slightly raised, smooth to granular, aerial mycelium same as surface; reverse colorless, pale gray to gray (near gray series 3 dc, 3 fe); no soluble pigment.

Glucose-Asparagine Agar - Growth moderate; yellow, gray to pink-gray (2 ic, near gray series 3 fe, 7 ih); appearing as isolated colonies; smooth, wrinkled to granular; aerial mycelium gray to pink-gray; reverse pink-gray (near gray series 7 ih); soluble pigment none to pale pink (4 ca).

Gordon and Smith's Tyrosine Agar - Growth moderate, brown (4 le); slightly raised, smooth to slightly granular; aerial mycelium sparse, white to pale gray (near gray series 3 dc); reverse brown (3 ic, 4 lc); soluble pigment yellowish brown (3 nc).

Casein Agar - Growth good; white, grayish yellow to lavender (2 gc, 4 ge); raised, smooth to wrinkled, aerial mycelium white; reverse yellowish to lavender (2 ga, 2 ic, 4 ge); soluble pigment yellowish brown (3 lc).

Bennett's Agar - Growth excellent; white, pale gray to gray (near gray series 3 dc, 3 fe); highly raised; smooth, slightly wrinkled to granular; aerial mycelium same as surface; reverse pink-gray to dark pink-gray (near gray series 7 ih, 7 ml); soluble pigment yellowish (2 ga).

Emerson's Agar - Growth good, tan to pink (near 3 gc, 5 ea); raised, slightly wrinkled, no aerial mycelium; reverse brown (3 ie); soluble pigment yellowish brown (3 lc).

Nutrient Agar - Growth moderate; white, pale pink-gray to pink-gray (near gray series 5 ic, 5 fe); appearing as isolated colonies, moderately raised, smooth to wrinkled; aerial mycelium same as surface; reverse yellowish to brown (2 ga, 2 ic, 4 ie); no soluble pigment.

Calcium Malate Agar - Growth scant, pale gray to gray (near gray series 3 dc, 3 fe); slightly raised, thin, smooth, aerial mycelium same as surface; reverse same as surface; no soluble pigment.

Potato Carrot Agar - Growth moderate, pink-gray to dark pink-gray (near gray series 5 ih, 5 ml), appearing as isolated colonies, slightly raised, smooth, aerial mycelium same as surface; reverse same as surface; no soluble pigment.

Tap Water Agar - Growth poor; pale gray, gray to pink-gray (near gray series 3 dc, 3 fe, 7 ih); thin, smooth; aerial mycelium same as surface; reverse pale gray to gray (near gray series 3 dc, 3 fe); no soluble pigment.

Gelatin Agar - Growth moderate to good; white to brown (3 le), but pale yellowish brown (3 gc) at edge; moderately raised; smooth, granular to slightly wrinkled; aerial mycelium white; reverse brown to dark brown (4 ie, 4 pi, 4 ni); no soluble pigment.

Starch Agar - Growth moderate to good; white, pale gray, gray, brown to dark brown (4 ie, 4 nl, near gray series 3 dc, 3 fe); moderately raised, smooth to granular; aerial mycelium same as surface; reverse brown to dark brown (4 lg, 4 li); soluble pigment yellowish (2 lc).

Morphological Properties - The morphological observations were made on oatmeal agar after 15 days of incubation: spore mass in Gray color-series; spore chains in Section Spirales, tightly coiled or slightly open or coiled into an irregular mass, of small diameter (3 - 4 μm), 3 - 8 turns per spore chain, may aggregate into a hygroscopic mass, 10 to 50 spores per spore chain; sporophores monopodially branched; spores short-rod shaped to rod shaped, sometimes globose, oval to elliptical, straight, but sometimes slightly curved, often with both ends not parallel to each other, 0.9 - 1.8 x 0.8 - 1.1 μm or 0.9 - 1.2 μm diam.; smooth to warty with a rugose surface, as revealed by scanning electron microscopy.

Biochemical Properties - Melanin not produced; hydrogen sulfide produced; gelatin liquefied; starch hydrolyzed; nitrate not reduced to nitrite; good growth but no decomposition on both cellulose broths; clearing but no coagulation on milk; casein digestion positive; tyrosine

digestion positive; calcium malate digestion negative. Carbohydrate utilization: Glucose, fructose, inositol, mannitol, raffinose, sucrose, and xylose utilized; arabinose and rhamnose not utilized.

Temperature Relations -

21°C	28°C	37°C	45°C
Moderate Growth	Excellent Growth	Good Growth	No Growth

Cell Wall Analysis - The whole-cell hydrolysates contained LL-diaminopimelic acid and galactose.

The culture N927-101SC50 is characterized by the gray spores in mass, the negative melanin reaction, the spiral spore chains, and the smooth to warty spores. The whole cell hydrolysates indicate the presence of LL-diaminopimelic acid and galactose. Glucose, fructose, inositol, mannitol, raffinose, sucrose, and xylose were utilized; but arabinose and rhamnose were not utilized. Therefore, the culture belongs to the genus Streptomyces.

The parent culture N927-101 resembles culture N927-101SC50 in all of the biochemical properties and temperature range except for minor differences in culture characteristics. On yeast extract-malt extract agar, culture N927-101 produced dark purple rather than yellow to dark brown colony reverse and purple rather than no soluble pigment. It sporulated better than N927-101SC50 on inorganic salts-starch agar, starch agar, and gelatin agar, but worse on nutrient agar. Although the black hygroscopic patches in culture N927-101SC50 were not as obvious as in culture N927-101 at 15 days of incubation, almost all of the colonies of both cultures have turned black on the following agars after six weeks of incubation: yeast extract-malt extract agar, oatmeal agar, inorganic salts-starch agar, Czapek-sucrose agar, Bennett's agar, potato carrot agar, and tap water agar. Thus, it is concluded that culture N927-101SC50, which is a natural isolate of culture N927-101, is the same as culture N927-101.

When culture N927-101SC50 was compared with FK-506 producing culture S. tsukubaensis BP-927 and FK-520 and FK-523 producing culture S. hygroscopicus subsp. yakushimaensis BP-928 (available from the Fermentation Research Institute in Japan), the differences are apparent (Table 1). In addition, culture S. tsukubaensis produced an orange tint of soluble pigment on yeast extract-malt extract agar, oatmeal agar, and glucose-asparagine agar; whereas S. hygroscopicus subsp. yakushimaensis produced no distinct soluble pigment. On yeast extract-malt extract agar, and inorganic salts-starch agar, the colony reverse was brown and gray-pink, respectively, for S. tsukubaensis, but was gray to dark gray and yellow-gray to black for S. hygroscopicus subsp. yakushimaensis.

When compared with known species of Streptomyces, the culture N927-101SC50 closely resembles Streptomyces braegensis Dietz NRRL 12567 (U.S. Patent 4,404,190, Sept. 13, 1983), S. braegensis Dietz subsp. japonicus Huang N617-29 (Rance, M. J. et al., J. Antibiot., 42, 206-217 (1989)), and S. nigrescens (Sveshnikova) Pridham et al. ATCC 23941 (Shirling, E.B. and D. Gottlieb, Int. J. Syst. Bacteriol, 18, 279-396 (1968)). These three cultures were grown for comparisons.

Culture N927-101SC50 is similar to S. nigrescens in most of the biochemical properties except that the former, but not the latter, produced hydrogen sulfide and showed a better growth on cellulose broth. S. nigrescens, unlike culture N927-101SC50, showed no distinct soluble pigment and no distinct pigment on colony reverse. The colors of colony reverse ranged from yellow, yellow-gray, and gray to sometimes black.

Culture N927-101SC50 is similar to S. braegensis subsp. japonicus in almost all of the biochemical properties except for its ability to produce hydrogen sulfide. Generally, S. braegensis subsp. japonicus did not produce distinct soluble pigment. Its colony reverse varied from yellow, gray-brown, and gray-yellow-brown to dark brown.

Culture N927-101SC50 has the same biochemical properties as S. braegensis except for its ability to produce hydrogen sulfide and better growth in cellulose broth. The colony reverse of the latter was pale pink, reddish pink, and reddish on glucose-asparagine agar, Bennett's agar, and starch agar, respectively; but on these three media the former had a pink-gray to brown colony reverse. On yeast extract-malt extract agar, inorganic salts-starch agar and casein agar, the former had a purple colony reverse; but the latter had a yellow-brown, pale gray to brown, and pale yellowish colony reverse, respectively. Culture N927-101SC50 produced pink-gray but not pale yellow-brown soluble pigment on oatmeal agar, and yellow brown to pink but not cream to yellow-brown soluble pigment on inorganic salts-starch agar.

On the basis of the above results, it is concluded that the culture N927-101SC50 represents a new subspecies of S. braegensis and is designated Streptomyces braegensis subsp. pulcherrimus Huang subsp. nov. The subspecific epithet (L. adj., pulcherrimus, prettiest) refers to the beauty of a purple pigment produced on some solid media and in ATCC 172 broth. It has been deposited at the American Type Culture Collection under the accession number 55150.

TABLE 1

Comparisons of N927-101SC50 with S. tsukubaensis and S. hygroscopicus subsp. yakushimaensis

Organism / Properties	N927-101SC50	S. tsukubaensis BP-927	S. hygroscopicus subsp. yakushimaensis BP-928
Compound produced	Compound of formula I	FK-506	FK-520 FK-523
Spore color in mass	Gray	Gray or Red	Gray
Spore chains	spiral	straight	spiral
Spore Surface	smooth to rugose	smooth	rugose
Growth in L & S cellulose broth	+	-	-
Degradation of tyrosine	+	-	+
Coagulation of milk	-	+	+
Utilization			
Arabinose	-	-	+
Fructose	+	-	+
Mannitol	+	-	+
Raffinose	+	-	-
Rhamnose	-	+	-

The Streptomyces culture used to produce the compound of formula I (e.g., N927-101 or N927-101SC50) is preferably grown at a temperature from about 20°C to about 40°C more preferably from about 24°C to about 36°C, under submerged conditions with agitation and aeration in a medium

consisting of a carbon source, a nitrogen source, one or more mineral salts containing trace elements such as iron, cobalt, copper, zinc etc., and one or more buffering agents. The compound can be recovered by extracting the whole broth with an organic solvent (e.g., n-butanol, methyl isobutyl ketone, ethyl acetate or chloroform), preferably at a pH of about 4.0 to about 8.0. Alternatively, after growth has been completed, the mycelium may be separated and extracted with an organic solvent (e.g., acetone or methanol) as described above, and the filtrate discarded. The solvent is concentrated to a thin syrup, triturated with an organic solvent (e.g., heptane) and then chromatographed (e.g., on silica gel) to obtain the pure compound.

The preferred sources of carbon in the nutrient medium are carbohydrates such as glucose, xylose, galactose, glycerin, starch, dextrin, maltose, rhamnose, raffinose, arabinose, mannose, and salicin. Other sources which may be included are corn steep liquor, grain solubles, fish meal and cotton seed meal.

The preferred sources of nitrogen are yeast extract, meat extract, fish meal peptone, gluten meal, cottonseed meal, soybean meal and other vegetable meals (partially or totally defatted), casein hydrolysates, yeast hydrolysates, corn steep liquor, dried yeast, wheat germ, feather meal, peanut powder, distillers solubles as well as inorganic and organic nitrogen compounds such as ammonium salts (e.g. ammonium nitrate, ammonium sulfate, ammonium phosphate, etc.), urea and amino acids (e.g., casamino acids).

The preferred buffering agents are calcium carbonate or phosphates. Other suitable buffering agents include morpholinoethanesulfonic acid (MES) and morpholinopropanesulfonic acid (MPS). Various nutrient materials may be used which inherently possess buffering properties.

The carbon and nitrogen sources, though advantageously employed in combination, need not be used in their pure form. Less pure materials, which contain traces of growth

factors and considerable quantities of mineral nutrients, are also suitable for use. When desired, there may be added to the medium mineral salts such as sodium or calcium carbonate, sodium or potassium phosphate, sodium or potassium chloride, sodium or potassium iodide, magnesium salts, copper salts, iron salts, zinc salts, cobalt salts, and the like. If necessary, especially when the culture medium foams, a defoaming agent, such as liquid paraffin, fatty oil, plant oil, mineral oil or silicone may be added.

Submerged aerobic cultural conditions are preferred for producing large amounts of the compound of the formula I. For the production of the compound of the formula I in small amounts, a shaking or surface culture in a flask or bottle is employed. When fermentation is carried out in large tanks, it is preferable to use the vegetative form of the organism for inoculation in the production tanks in order to avoid growth lag in the process of production of the compound of the formula I. Accordingly, it is desirable first to produce a vegetative inoculum of the organism by inoculating a relatively small quantity of culture medium with spores of mycelia of the organism produced in a "slant" and culturing said inoculated medium, also called the "seed medium", and then to transfer the cultured vegetative inoculum aseptically to large tanks. The fermentation medium, in which the vegetative inoculum is produced, may be substantially the same as or different from the medium utilized for the production of the compound of the formula I and is generally autoclaved to sterilize the medium prior to inoculation. The pH of the medium is generally adjusted to below 8.0 prior to the autoclaving step by suitable addition of an acid or base, preferably in the form of a buffering solution.

Agitation and aeration of the culture mixture may be accomplished in a variety of ways. Agitation may be provided by a propeller or similar mechanical agitation equipment, by revolving or shaking the fermentor by various pumping equipment or by the passage of sterile air through

the medium. Aeration may be effected by passing sterile air through the fermentation mixture.

The fermentation is usually conducted at a temperature between about 20°C and about 40°C, preferably about 24°C to about 36°C, for a period of about 50 hours to about 150 hours, which may be varied according to fermentation conditions and scales. Preferably, the production cultures are incubated for about 96 hours at 27°C on a rotary shaker operating at 220 rpm, wherein the pH of the fermentation medium is preferably maintained at a pH of about 4.0 to about 8.0, more preferably about 6.0 to about 8.0, to harvest.

Culturing/production media that may be used for carrying out the fermentation include the following:

Medium A: glycerol, corn starch, cerelose, cotton seed meal, torula yeast, corn steep liquor, calcium carbonate and cobalt chloride, in preferred weight percentages of the fermentation medium, respectively, of: 1.0%, 1.0%, 0.5%, 1.0%, 0.5%, 0.5%, 0.2%, and 0.0005% and 0.2% (volume/volume) of an antifoam agent such as P-2000 (Dow Corning (Trademark)). The pH of this medium is generally adjusted to about 6.3 to about 6.5 prior to autoclaving and inoculation.

Medium B: corn starch, corn steep liquor, torula yeast, magnesium sulfate, potassium dibasic phosphate, cobalt chloride and calcium carbonate, in preferred weight percentages of the fermentation medium, respectively, of: 4.5%, 1.0%, 1.0%, 0.01%, 0.01% and 0.0001%, and 0.2% (volume/volume) of an antifoam agent such as P-2000 (Dow Corning (trademark)). The pH of this medium is generally adjusted to about 7.0 to about 7.2 prior to autoclaving and inoculation.

Medium C: cerelose, soybean meal, ammonium sulfate, potassium dibasic phosphate, calcium carbonate, NZ amine YTT (Sheffield (trademark)) and cobalt chloride in preferred weight percentages of the fermentation medium, respectively, of: 2.0%, 3.0%, 0.5%, 0.5%, 0.3%, 0.5% and 0.0005% and 0.2%

(volume/volume) of an antifoam agent such as P-2000 (Dow Corning (trademark)). The pH of this medium is generally adjusted to about 7.0 to about 7.2 prior to autoclaving and inoculation.

The produced compound of the formula I can be recovered from the culture medium by conventional means which are commonly used for the recovery of the other known biologically active substances. The compound of the formula I produced is found in the cultured mycelium and filtrate, and accordingly can be isolated and purified from the mycelium and the filtrate. The mycelium can be separated, by filtering or centrifuging the cultured broth. The mycelium can then be extracted with a conventional solvent, such as methanol or acetone, separated and concentrated. Treatment of the concentrate with a conventional resin (e.g., anion or cation exchange resin, non-ionic adsorption resin, etc.), treatment with a conventional adsorbent (e.g., activated charcoal, silic acid, silica gel, cellulose, florisil, alumina, etc.), crystallization, and recrystallization are then performed to recover the desired compound.

In a preferred method for preparing the compound of the formula I, inoculum is prepared by scraping vegetative cells from slants or Roux bottles inoculated with the N927-101 culture. A solid medium suitable for initial growth on slants and Roux bottles is ATCC medium 172 described below:

Ingredient	Grams/liter
Glucose	10
Soluble starch	20
Yeast extract	5
NZ Amine A	5
Calcium carbonate	1
Agar	20

Distilled water to 1000 ml;
pH to 7.0 with KOH;

Vegetative cells from slants are used to inoculate either shake flasks or inoculum tanks; or alternatively the

inoculum tanks are inoculated from shake flasks. In shake flasks, growth will generally have reached its maximum in 48 to 96 hours whereas in the inoculum tanks growth will usually be at the most favorable period in 72 to 120 hours. A fermenter is inoculated with vegetative broth from the inoculum flasks or tank under completely aseptic conditions and fermented for a period of 72 to 120 hours. Aeration is maintained in the shake flask by agitation on a shaker or in tanks by forcing sterile air through a sparger at the rate of 1/2 to 2 volume of air per volume of broth per minute. The speed of agitation (stirring) depends upon the type of agitator employed; a shake flask is usually run at 150 to 200 cycles per minute (cpm) and a fermenter at 300 to 1700 revolutions per minute (rpm). Sterility must be maintained at all times. The temperature is regulated between 24°C and 36°C. Foaming during the fermentation can be controlled with sterile antifoams such as refined soybean oil, or other suitable antifoaming agents added to the makeup or to the fermenter aseptically as needed after inoculation.

Shake flasks are prepared using one of the following media:

ATCC 172	Grams/Liter
Glucose	10
Soluble starch	20
Yeast extract	5
NZ Amine A (Trademark of Quest International Norwich, New York)	5
Calcium Carbonate	1
	pH 7.0 ~ 7.1

FK-506S	Grams/Liter
Glycerol	10
Corn starch	10
Cerelose	5
Cotton seed meal	10
Torula yeast	5
Corn steep liquor	5
Calcium Carbonate	2
Cobalt Chloride	0.005
	pH to 6.3 ~ 6.5

JDYTT	Grams/Liter
Cerelose	10
Corn starch	5
NZ Amine YTT (Trademark of Quest International Norwich, New York)	5
Corn steep liquor	5
Calcium carbonate	3
Cobalt chloride	0.005
	pH to 6.5 ~ 6.7

One hundred ml of medium is distributed into 300 ml shake flasks and sterilized at 120°C and 1.06 kg/cm^2 for 30 minutes. After cooling, the medium is inoculated with a vegetative cell suspension from the N927-101 slant culture grown on ATCC medium 172 in agar. The flasks are shaken at 28°C on a shaker having a displacement of 2.5-3.8 cm to 5.0-

7.3 cm and 150 to 200 rpm for 2 to 4 days. Ten ml may be used to inoculate one 2.8 liter Fernbach flask containing 1 liter of media or 1 flask may be used to inoculate a 5 liter fermentation vessel containing 3 liters of 1 of the following media:

FK-506-FM	Grams/ Liter	ATCC 172	Grams/ Liter
Corn starch	45	Glucose	10
Corn steep liquor	10	Soluble starch	20
Torula yeast	10	Yeast extract	5
Magnesium sulfate	0.1	NZ Amine A	5
Potassium dibasic phosphate	0.1	Calcium carbonate	1
Cobalt chloride	0.001		
Calcium carbonate	2		

pH to 7.0 ~ 7.2

One ml of P-2000 is added as an antifoaming agent to the jar fermenters only, then the vessels are sealed and sterilized at 120°C and 1.06 kg/cm^2 for 60 to 90 minutes. The jars are inoculated with one (about 3% inoculum) flask, fermented for 72 to 120 hours at 27°C and stirred at 1700 rpm with an air rate of one volume of air per volume of liquid per minute. The shake flasks (Fernbachs) are run at 150 ~ 200 cpm on a shaker at 28°C and fermented for 72 to 120 hours. The fermenters are stopped and the contents extracted twice with one third to one half volume of a solvent such as methyl isobutyl ketone or n-butanol. The solvent layer is separated by aspiration or centrifugation, sparkled, and concentrated in vacuo to a viscous oil.

The bioactivity of the broth and subsequent recovery streams can be followed by HPLC or by using a strain of a filamentous fungi, e.g., Byssochlamys fulva. The components in the broth and recovery streams can also be visualized by chromatography on Analtech silica gel FG (trademark) plates using neat ethyl acetate or chloroform-methanol 10:1 as the eluant. The developed plates are viewed under ultraviolet light at 254 nm or are sprayed with vanillin reagent (e.g.,

3 g vanillin in 75 ml of ethanol and 25 ml 85% phosphoric acid) and heated to 80°C. The compound appears as a gray blue spot. Extracts of the broth may be run in a Waters (trademark) analytical HPLC using a Zorbax CN (trademark) column and an isocratic system such as H_2O-acetonitrile 55:45.

Larger scale fermentations may be carried out by preparing shake flasks containing one liter of compound of formula I inoculum medium. The shake flask inoculum is fermented for 2 to 4 days at 28°C, and then used to inoculate a 50-gallon fermenter containing 25 gallons of production medium. The broth is harvested after running 3 to 5 days. The whole broth is extracted with 1/3 volume of methyl isobutyl ketone (MIBK) at natural pH, separated on an Alpha De Laval (trademark) separator and the solvent phase concentrated in vacuo to an oil. The term natural pH refers to the pH of the fermentation when stopped, or, if the pH is controlled, the pH at which the fermentation was run or maintained. The oil thus obtained may be processed as described below in Example 1.

Larger fermenters can be staged. A 100-gallon charge of compound of formula I seed medium is inoculated by side arm flask, run approximately 48 hours, then pumped aseptically into 1000 gallons of production medium.

The compound of the formula I inhibits the rotamase activity of human FK-binding protein. Functional biological activity in a human mixed lymphocyte reaction (MLR) may be demonstrated by standard procedures well known to those skilled in the art.

The immunosuppressant effect of the compound of the formula I may also be demonstrated by the following assay which measures the proliferation of mouse T lymphocytes stimulated with the combination of ionomycin plus PMA (phorbol myristate acetate). A positive sample in this assay will inhibit T-cell proliferation, as indicated by reduced tritiated thymidine uptake.

Assay

Spleens from C57BI/6 mice are taken under sterile conditions and gently dissociated in ice-cold RPMI 1640 (trademark) culture medium (GIBCO (trademark)) supplemented with 10% heat-inactivated fetal calf serum (GIBCO (trademark)). Cells are pelleted by centrifugation at 1500 rpm for 8 minutes. Contaminating red cells are removed by treating the pellet with ammonium chloride lysing buffer (GIBCO (trademark)) for 2 minutes at 4°C. Cold medium is added and cells are again centrifuged at 1500 rpm for 8 minutes. T lymphocytes are then isolated by separation of the cell suspension on nylon wool columns as follows: Nylon wool columns are prepared by packing approximately 4 grams of washed and dried nylon wool into 20 ml plastic syringes. The columns are sterilized by autoclaving at 120°C for 30 minutes. Nylon wool columns are wetted with warm (37°C) culture medium and rinsed with the same medium. Washed spleen cells resuspended in warm medium are slowly applied to the nylon wool. The columns are then incubated in an upright position at 37°C for 1 hour. Non-adherent T lymphocytes are eluted from the columns with warn culture medium and the cell suspensions are spun as above.

Purified T lymphocytes are resuspended at 10^6 cells/ml in complete culture medium composed of RPMI 1640 (trademark) medium with 10% heat-inactivated fetal calf serum, 100 mM glutamine, 1mM sodium pyruvate, 20 μm 2-mercaptoethanol and 50 μg/ml gentamicin. Ionomycin is added at 250 ng/ml and PMA at 10 ng/ml. The cell suspension is immediately distributed into 96-well flat-bottom microculture plates (Costar (trademark)) at 100 μl/well. Control (cyclosporin) medium or sample to be tested are then added in triplicate wells at 10 μl/well. The culture plates are then incubated at 37°C in a humidified atmosphere of 5% CO_2-95% air for 44 hours. The proliferation of T lymphocytes is assessed by measurement of tritiated thymidine incorporation. After 44 hours of culturing, the cells are pulse-labelled with 2 Ci/well of tritiated thymidine (New England Nuclear

(trademark)). After another 4 hours of incubation, cultures are harvested on glass fiber filters using a multiple sample harvester. Radioactivity of filter discs corresponding to individual wells is measured by standard liquid scintillation counting method (Beta plate counter). Mean counts per minute of replicate wells are calculated and the results expressed as percent inhibition of tritiated thymidine uptake (proliferation) as follows:

$$\% \text{ Inhibition} = 100 - \frac{\text{Mean cpm sample tested}}{\text{Mean cpm control medium}} \times 100.$$

The compound of the formula I has immunosuppressive activity and is therefore useful for the treatment and prevention of the transplantation rejection of organs or tissues such as heart, kidney, liver, medulla ossium, skin, etc., graft-versus-host diseases by medulla ossium transplantation, autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, Hashimoto's thyroiditis, multiple sclerosis, myasthenia gravis and type I diabetes. The compound also has antimicrobial activity and is useful in treating infectious diseases caused by pathogenic filamentus fungal organisms.

The pharmaceutical compositions of the present invention can be used in the form of a pharmaceutical preparation, for example, in solid, semi-solid or liquid form, which contains a compound of the formula I as an active ingredient, in admixture with an organic or inorganic carrier or excipient suitable for external, enteral or parenteral applications. The active ingredient may be compounded, for example, with the usual non-toxic, pharmaceutically acceptable carriers for tablets, pellets, capsules, suppositories, solutions, emulsions, suspensions, and any other form suitable for use. The carriers which can be used are water, glucose, lactose, gum acacia, gelatin, mannitol, starch paste, magnesium trisilicate, talc, corn starch, keratin, colloidal silica, potato starch, urea and other carriers suitable for use in manufacturing preparations, in solid, semi-solid, or liquid form, and in

addition auxiliary, stabilizing, thickening and coloring agents and perfumes may be used. For applying such pharmaceutical compositions to a human, it is preferable to apply the compositions by parenteral or oral administration.

While the dosage of therapeutically effective amount of the compound of formula I depends upon the age and condition of each individual patient to be treated, a daily dose (calculated on the basis of a 70 kg man) of about 0.01 to about 1000 mg, preferably about 0.1 to about 500 mg and more preferably about 0.5 to about 100 mg of the active ingredient is generally given for treating transplantation rejection or the diseases referred to above, and an average single dose of about 0.5 mg, 1 mg, 5 mg, 10 mg, 50 mg, 100 mg, 250 mg or 500 mg is generally administered.

The following examples are given for the purpose of illustrating the present invention and should not be construed as being limitations on its scope.

Example 1

Fermentation of N927-101

The organism N927-101 was isolated from a soil sample collected in Misumitown, Yamaguchi Prefecture, Japan.

A. Microtiter Fermentation

A sample of N927-101 colony on an agar-plate was inoculated into a seed medium W (2.5 ml) containing glucose (1 g), dextrin, polypeptone (5 g/l), yeast extract (5 g/l), beef extract (3 g/l) and $CaCO_3$ (4 g/l) in a 24-well microtiter plate and cultured at 28°C for 3 days on a rotary shaker with 7-cm throw at 200 rpm. The seed culture (0.15-0.3 ml) thus obtained was inoculated to a production medium (3 ml) containing glucose (10 g/l), corn starch (20 (g/l), NZ amine type-A (5 g/l), yeast extract 5 (g/l), wheat embryo (5 g/l), $COCl_2 \cdot H_2O$ (0.001 (g/l) and $CaCO_3$ (4 g/l) in a 24-well microtiter plate and cultured under the same conditions as the above seed culture except that the fermentation was 4 days.

The fermentation broth thus obtained was applied to a TLC (thin layer chromatography) plate (Kieselgel $60F_{254}$, E.

Merck (trademark) 20x20 cm) and developed by $CHCl_3$: CH_3OH (9:1). After the solvent was removed, the TLC plate was placed in a bioplate (28x44 cm) and overlayed by 300 ml of malt yeast agar (malt extract 20 g, yeast extract 4 g, agar 20 g in 1,000 ml) seeded with Byssochlamys fulva. The bioplate was incubated at 28°C for 48 hours. At least two compounds were detected as the inhibition zones against the test organism with R_f values of 0.30 and 0.55.

B. Flask Fermentation

In order to determine whether the compounds produced by the N927-101 colony where the same as known compounds, flask fermentation was carried out. A seed culture (100 ml) inoculated with 0.5 ml of frozen seed-culture from the microtiter fermentation was fermented as described above and then 5 ml were used to prepare 100 ml of a main culture which was fermented as described above. 150 ml of the resulting fermentation broth was extracted with 100 ml of ethyl acetate (EtOAc). The EtOAc solution was concentrated to dryness. The residue was dissolved in 2 ml of acetone and submitted to TLC-bioautography. The TLC-bioautography with Byssochlamys fulva indicated that two FK-related compounds were reproduced. One of them having the Rf value of 0.55 was identical to FK-520 based on the HPLC analysis (Zorbax CN, Dupont (trademark) column: 4.6 mm x 25 cm; eluent: 55% water/45% acetonitrile; flow rate: 1 ml/min; detection: UV 214 nm). Another compound with the Rf value of 0.30 was novel.

C. Mini-jar Fermentation

In order to isolate the novel compound and determine the structure, mini-jar fermentation was carried out. A first seed culture was prepared by inoculating 100 ml of medium with 5 ml of frozen seed culture from the shake-flask fermentation. The medium (WGB medium) comprised water, glucose (20 g/l), polypeptone (5 g/l), yeast extract (5 g/l), beef extract (3 g/l), wheat gluten (5 g/l), blood meal (3 g/l), and $CaCO_3$ (4 g/l). The culture was maintained at 28°C for four days with rotation at 200 rpm.

Second seed cultures were prepared by inoculating 150 ml of WGB medium with 7.5 ml of the fermented first seed culture. The culture was maintained at 28°C for three days with rotation at 200 rpm and aeration at 1.0 volume/volume/minute.

Main cultures were prepared in five 6-liter mini-jars by inoculating 3 liters of medium in each jar with 150 ml of the second seed culture. The medium (IT-2 medium) comprised glucose (10 g/l), dextrin (20 g/l), wheat gluten (10 g/l), corn steep liquor (5 g/l), polypeptone (1 g/l), $(NH_4)_2SO_4$ (1 g/l), $CaCO_3$ (4 g/l) and $COCl_2 \cdot 6H_2O$ (0.001 g/l).

The whole mini-jar fermentation broth (15 l) was freeze-dried and the solid (397 g) was extracted with two 5-liter portions of 70% aqueous acetone and concentrated to 2.5 l of aqueous suspension. The suspension was extracted with two 2-liter portions of methyl isobutyl ketone and the combined extract was concentrated to oily residue. A small amount of ether (100 ml) was added to the oily residue to give a precipitate. After collecting the precipitate (2.8 g), the ether solution was concentrated and 100 ml of n-hexane was added to yield 1.7 g of gum-solid. The 2.8 and 1.7 g gum solids were combined and applied to a 120 g fine mesh silica gel column which was developed with 1 L of 3:1 hexanes/ethyl acetate, followed by 1 L of 2:1 hexanes/ethyl acetate, 1 L of 1:1 hexanes/ethyl acetate, 1 L of 2:1 ethyl acetate/hexanes and finally by 2 L of ethyl acetate to yield 25 mg of FK-520 and 125 mg of a mixture of FK-520 and a novel compound. This mixture was applied to a 10 g fine mesh silica gel column which was eluted with 200 ml of chloroform followed by 200 ml of chloroform with 2% methanol, 200 ml of chloroform with 5% methanol, and finally 100 ml of chloroform with 10% methanol to yield 10 mg of the new compound. This was further purified on a 1 g C_{18} column developed with methanol and water in a ratio of 4/1 to yield 4 mg of the novel compound later found to have the formula I.

Example 2

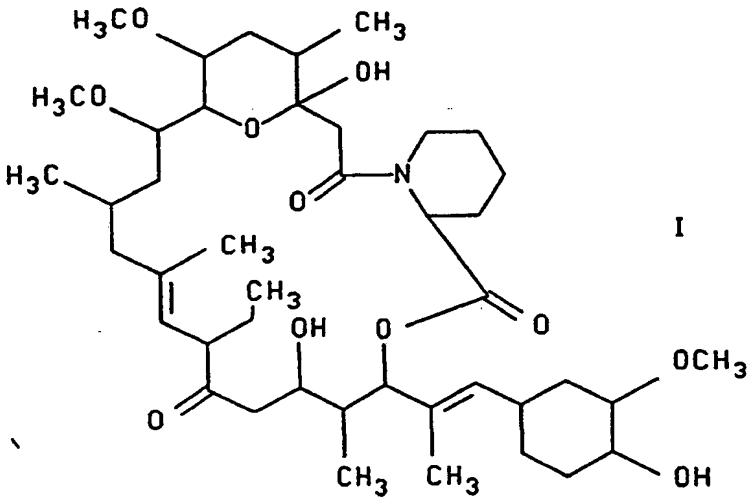
Large Scale Flask Fermentation

Shake flasks containing 80 ml of ATCC 172 medium were inoculated and fermented for 3 to 4 days at 28°C, then 10 ml was used to inoculate a 2.8 liter Fernbach flask containing one liter of ATCC 172 medium. The fermenter, after running 3 to 5 days, was harvested.

47 liters of N927-101 broth was extracted with methyl isobutyl ketone (MIBK) at natural pH. The MIBK phase was separated and concentrated in vacuo to an oil. The oil was defatted using acetonitrile and hexanes to give 4.0 g of a dark viscous liquid. This residue was chromatographed on 128 g of fine mesh silica gel which was pre-equilibrated with hexanes and ethyl acetate (1:1). The column was developed with 1 L of 1:1 ethyl acetate/hexanes, followed by 1 L of 2:1 ethyl acetate/hexanes, 1 L of 4:1 ethyl acetate/hexanes and finally by 1.5 L of ethyl acetate to yield 290 mg of a yellow oil. This mixture was then chromatographed on a second fine mesh silica gel column which was pre-equilibrated with chloroform and developed with 200 ml of chloroform, followed by 500 ml of chloroform with 1% methanol and then with 500 ml of chloroform with 2% methanol to yield 125 mg of an oil. This oil was chromatographed on Baker (trademark) C_{18} bulk packing for flash chromatography using methanol and water (3:1) to give 42 mg of product. Proton NMR and carbon NMR (see Figures 1 and 2) indicated the chemical structure of the product to be as shown in formula I. The molecular weight of 777, as determined by FAB (fast atom bombardment) mass spectroscopy, is consistent with the formula I.

CLAIMS

1. A process for preparing a compound of the formula



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comprising fermenting under aerobic conditions a compound of the formula I producing strain of bacteria in an aqueous medium containing a carbon source and a nitrogen source.

2. A process according to claim 1 comprising fermenting an organism having the characteristics of Streptomyces braegensis subsp. pulcherrimus Huang subsp. nov., ATCC 55150, or fermenting ATCC 55150, in either case under aerobic conditions in an aqueous medium containing a carbon source and a nitrogen source.

3. A process according to claim 1 or 2, wherein said fermentation is conducted at a pH between about 4.0 and about 8.0.

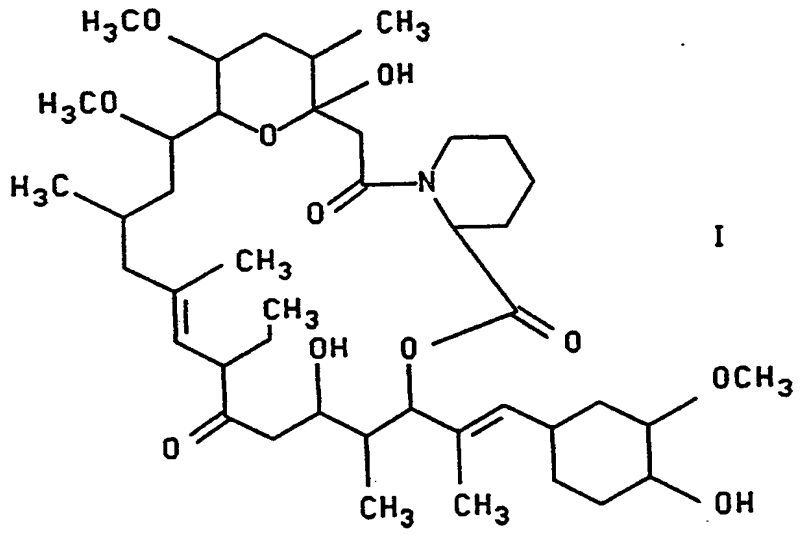
4. A process according to claim 1 or 2, further comprising recovering said compound of formula I by extracting the fermentation broth with an organic solvent at a pH of about 4.0 to about 8.0.

5. A process according to claim 1 or 2, further comprising recovering said compound of formula I by separating the mycelium and extracting it with an organic solvent at a pH of about 4.0 to about 8.0.

6. A process according to claim 1, wherein said fermentation is conducted at a temperature from about 20°C to about 40°C, for a period of time from about 50 hours to about 150 hours.

7. A process according to claim 9, wherein said fermentation is conducted at a temperature from about 24°C to about 36°C under submerged conditions with agitation and aeration on a medium consisting of a carbon source and a nitrogen source, one or more mineral salts containing trace metals and one or more buffering agents.

8. A biologically pure culture having the characteristics of Streptomyces braegensis subsp. pulcherrimus Huang subsp. nov., ATCC 55150, and any mutant or transformant thereof capable of producing the compound of the formula



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9. ATCC 55150, a biologically pure culture according to claim 14.

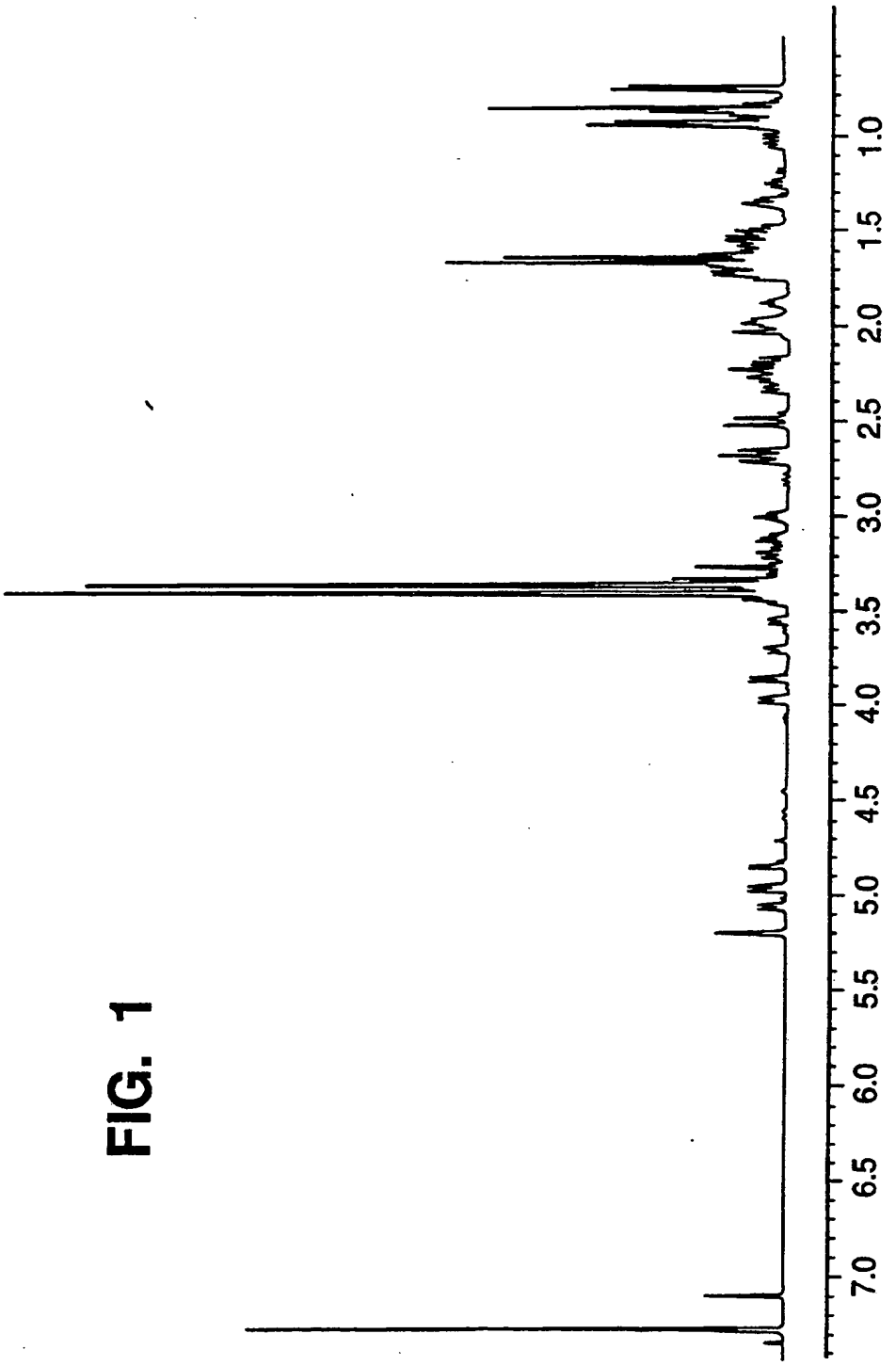
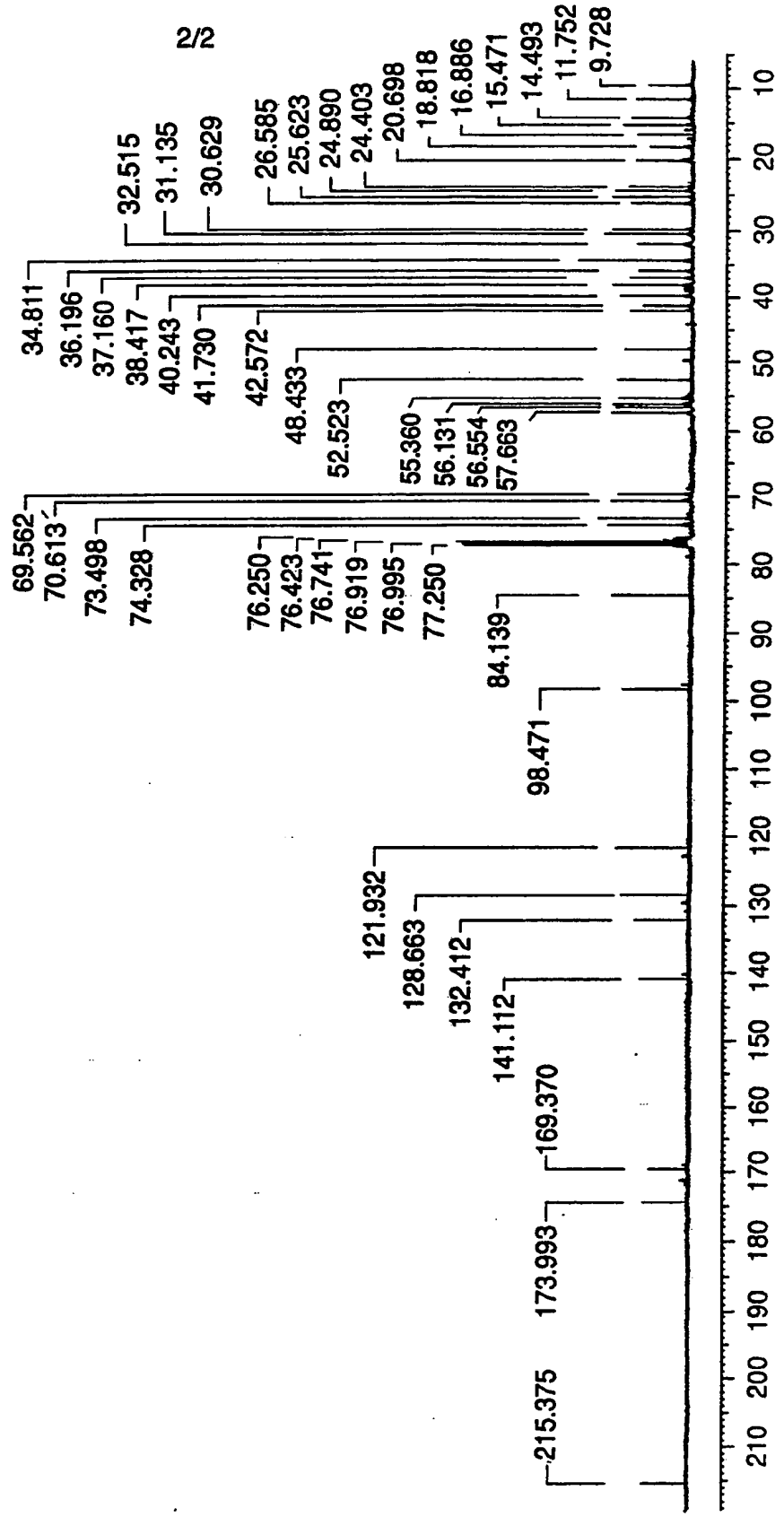


FIG. 1

FIG. 2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56.554
57.663
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98.471
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141.112
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173.993
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INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/02324

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C07D498/18; C12P17/18; C12N1/20; //(C12N1/20 C12R1:465)

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12P ; C07D ; C07H

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
A	WO,A,9 102 736 (FISONS PLC AND FUJIKAWA PHARMACEUTICAL COMPANY LIMITED) 7 March 1991 see page 2, line 1 - page 4, line 27 ---	1-9
A	EP,A,0 406 791 (FUJISAWA PHARMACEUTICAL CO. LTD.) 9 January 1991 see page 3, line 1 - page 4, line 7 ---	1-9
A	EP,A,0 402 931 (SANDOZ LTD.) 19 December 1990 see page 3, line 1 - line 50 see page 8, line 22 - line 29 ---	1-9
A	EP,A,0 323 042 (FISONS PLC) 5 July 1989 see page 3, line 1 - line 49 see page 4, line 28 - line 39 ---	1-9

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
24 JULY 1992	05.08.92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	KORSNER S.E.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9202324 SA 59909

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 24/07/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9102736	07-03-91	AU-A-	6286690	03-04-91
		CN-A-	1049503	27-02-91
		EP-A-	0487593	03-06-92
EP-A-0406791	09-01-91	AU-A-	5864290	24-01-91
		CA-A-	2020431	06-01-91
		CN-A-	1048496	16-01-91
		JP-A-	3128320	31-05-91
EP-A-0402931	19-12-90	AU-A-	5927790	08-01-91
		CA-A-	2032159	15-12-90
		WO-A-	9015805	27-12-90
		JP-T-	4500372	23-01-92
EP-A-0323042	05-07-89	AU-A-	2822889	05-07-89
		EP-A-	0346427	20-12-89
		WO-A-	8905304	15-06-89
		JP-T-	2502463	09-08-90